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Intratumoral heterogeneity of *KRAS* mutation is rare in non-small-cell lung cancer

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ABSTRACT

Background: Several lines of evidence indicate that mutational activation of KRAS is an early event in the carcinogenesis of non-small cell lung cancer (NSCLC). Nonetheless, previous studies report high frequencies of divergent *KRAS* mutational status between primary NSCLC and corresponding metastases. This suggests heterogeneity of the primary tumor in respect to its *KRAS* status. We therefore aimed to examine the frequency and the extent of such intratumoral heterogeneity.
Methods: 40 NSCLC were examined for intratumoral heterogeneity of *KRAS* mutation (20 adenocarcinomas, 10 squamous cell carcinomas and 10 large cell carcinomas). Three to eight different tumor areas were analyzed for *KRAS* mutation and up to four corresponding lymph node metastases were included for analysis in nineteen cases. A combination of different methods for screening of heterogeneity and its validation were used including direct sequencing, laser-capture microdissection for tumor cell enrichment and the very sensitive ARMS/S method.
Results: Mutations of *KRAS* were found in 13/30 adenocarcinomas and large cell carcinomas. No mutations were detected in 10 squamous cell carcinomas. Four cases showed heterogeneous *KRAS* results by direct sequencing. More sensitive methods for *KRAS* mutation analysis revealed false negative results due to admixture of non-neoplastic cells in all of these samples. Intratumoral heterogeneity of *KRAS* mutational status was therefore confirmed in none of the analyzed cases. In addition, identical *KRAS* mutations were present in the primary tumor and the corresponding lymph node metastases in 19 cases examined.
Conclusions: Intratumoral heterogeneity of *KRAS* mutational status is rare in NSCLC but highly sensitive tools are required to reliably identify these mutations. This finding is in line with the hypothesis that oncogenic activation of KRAS is an early event and a bona fide "driver mutation" in NSCLC. Furthermore, future therapies targeting KRAS will not be limited by intratumoral heterogeneity.

Introduction

Lung cancer is one of the most common human malignant diseases and causes most cancer related deaths worldwide (Jemal et al., 2009; Molina et al., 2008). About 85% of these tumors are non-small cell lung cancers (NSCLC) (WHO, 2004). Somatic mutation of the *KRAS* gene is one of the most frequent genetic alterations in NSCLC. While rare in squamous cell carcinomas, adenocarcinomas of the lung harbor *KRAS* mutations with a frequency of 15% to 57% in patients from United States and Europe (Graziano et al., 1999; Husgafvel-Pursiainen et al., 1993; Keohavong et al., 1996; Marks et al., 2008; Rodenhuis et al., 1987). Since the initial discovery of the *KRAS* gene 30 years ago, the significance of its mutations in lung cancer has been extensively investigated. KRAS is an important effector of the intracellular growth factor receptor signaling pathway. Activating *KRAS* mutation renders the tumor cell to become independent from external mitogenic signals and is believed to be one of the major events in carcinogenesis (Hanahan and Weinberg, 2000; Riely et al., 2009; Rodenhuis et al., 1987; Santos et al., 1984).

KRAS attracts significant attention as a target for anti-cancer therapy, but attempts to develop direct inhibitors of mutant KRAS have been unsuccessful so far (reviewed in (Gysin et al., 2011)). In recent years *KRAS* mutation in NSCLC also gained much attention since KRAS is a downstream effector of EGFR. With the introduction of EGFR targeting drugs such as EGFR tyrosine kinase inhibitors (TKI; erlotinib, gefitinib) and monoclonal antibodies directed against EGFR (cetuximab, panitumumab), *KRAS* mutations have been investigated as negative predictors of benefit from these agents. It was hypothesized that EGFR targeting drugs would be ineffective in controlling tumors with constitutively activated KRAS. However, in NSCLC no clear role for *KRAS* mutation as a negative predictor of response neither to EGFR TKIs nor to monoclonal antibodies emerged (Langer, 2011; O'Byrne et al., 2011; Riely et al., 2009; Roberts et al., 2010). This is in contrast to colorectal cancer, where *KRAS* mutation is now used to

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identify patients who will not benefit from EGFR targeting therapy (Lievre et al., 2006; Van Cutsem et al., 2009). Currently it is suggested to use *KRAS* mutations to exclude targetable genetic aberrations in NSCLC when performing a multistep molecular testing procedure for predictive markers (Bronte et al., 2010; Horn and Pao, 2009; Roberts et al., 2010). *EGFR* mutations or *EML4-ALK* fusions can be excluded in *KRAS* mutated adenocarcinomas as aberrations of these genes occur mutually exclusive.

NSCLC is often a heterogeneous disease, both on the morphological and the molecular level (Balsara and Testa, 2002; Carey et al., 1990; Forgacs et al., 2001). However, *KRAS* gene mutations are thought to occur early in the carcinogenesis of adenocarcinomas of the lung, as they are also found in precursor lesions such as atypical adenomatous hyperplasia (Ohshima et al., 1994; Westra et al., 1996, 1993; WHO, 2004). In addition, self-sufficiency in growth signals as acquired by *KRAS* mutation is believed to be one of the crucial steps during cell transformation (Hanahan and Weinberg, 2000). Reports on divergent *KRAS* mutational status between a primary lung carcinoma and its metastases are therefore puzzling. Nonetheless, several studies report such a divergence of the *KRAS* mutational status between primary tumor and metastases in a substantial fraction of NSCLC (Badalian et al., 2007; Cortot et al., 2010; Kalikaki et al., 2008; Schmid et al., 2009; Sun et al., 2011). This finding implicates heterogeneity of *KRAS* mutational status within the invasive tumor and indicates that *KRAS* mutation is a late event in the carcinogenesis of these tumors. Furthermore such heterogeneity would have major implications for future development of KRAS targeting therapies. In order to examine the frequency and extent of such heterogeneity, we analyzed neoplastic tissue from 45 NSCLC patients including 5 tumors of combined histologic type for intratumoral heterogeneity of *KRAS* mutation.

Materials and methods

Tissues and testing algorithm

Formalin-fixed, paraffin embedded tissue samples from 40 NSCLC patients who had received surgical treatment at the University Medical Center Hamburg-Eppendorf were examined. Patients were selected for this study if at least 4 different tumor containing paraffin blocks (primary tumor and/or lymph node metastases) had been retained. Among 161 cases that met this criterion in our database, 20 adenocarcinomas (cases 1 to 20), 10 squamous cell carcinomas (cases 21 to 30) and 10 undifferentiated large cell carcinomas (cases 31 to 40) without previous TKI treatment were randomly selected. Of the 20 adenocarcinomas 14 (70%) are of mixed subtype. These tumors show at least two different histologic subtypes such as acinar, papillary, bronchioloalveolar, mucinous or solid patterns. Initially *KRAS* mutation was evaluated with the ARMS/S method in one tumor sample per case. Screening of heterogeneity was then performed by direct sequencing of DNA. For each tumor 4 different samples were analyzed. Each sample was taken from a different tumor containing paraffin block. In 19 cases with available lymph node metastases 3 samples of the primary tumor and 1 sample of lymph node metastasis were analyzed. In case of *KRAS* mutation up to 4 additional primary tumor and/or lymph node blocks were analyzed to exclude heterogeneity of *KRAS* mutational status. Overall 181 different paraffin blocks (up to 8 different tumor and lymph node tissue blocks per case) were used for heterogeneity screening. Validation of heterogeneous *KRAS* results was then performed by laser-capture microdissection followed by sequencing and ARMS/S. In addition, five combined lung cancers including three adenosquamous, one combined small cell and one pleomorphic carcinoma (cases 41 to 45) were selected. *KRAS* mutation analysis in these cases was performed by direct sequencing after laser-capture microdissection of cells representing the divergent histologic tumor areas.

DNA extraction

From each paraffin block one representative area containing at least 60% tumor cells was selected by a pathologist and one tissue core (diameter 0.6 mm) was removed for DNA extraction using a home-made semi-automated tissue microarrayer (Sauter et al., 2003). Laser-capture microdissection of tumor tissue was performed by a pathologist using a PALM Micro Beam Laser System (Zeiss MicroImaging, Munich, Germany). A minimum of 1000 tumor cells with a purity of >90% was collected for subsequent DNA extraction. Before DNA extraction, paraffin was removed with xylene and ethanol. After digestion of the tissue with proteinase K at 56 °C overnight, DNA was isolated with the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantity and quality of DNA was evaluated using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, USA).

KRAS mutation analysis by ARMS/S

Template DNA was analyzed for a set of seven *KRAS* point mutations in codons 12 and 13 with the TheraScreen *KRAS* Mutation Detection kit (DxS Ltd., Manchester, UK) using 70 ng of genomic DNA per reaction. This method is based on an amplification refractory mutation system (ARMS) which incorporates a unique bifunctional fluorescent primer/probe molecule (Scorpion) and allows the detection of 1% mutant allele. Reactions were performed on a LightCycler 480 real-time PCR instrument (Roche Diagnostics, Mannheim, Germany) and analyzed with LightCycler Adapt software v1.1 (Roche Diagnostics) as described by the manufacturer.

KRAS mutation analysis by direct sequencing

One hundred nanograms of template DNA were PCR amplified using 10 pmol each of forward and reverse *KRAS* exon 2 primers (forward 5′-GCCTGCTGAAAATGACTGAA-3′ and reverse 5′-AGAATGGTCCTGCA CCAGTAA-3′) and AmpliTaq Gold PCR mastermix (Applied Biosystems, Darmstadt, Germany) in a 25 µl reaction. PCR was performed on a BioRad C1000 thermocycler (20 seconds at 95 °C, 20 seconds at 55 ° C and 40 seconds at 72 °C, 40 cycles). The quality of PCR products (167 bp) was confirmed by capillary electrophoresis on a QIAxcel system (Qiagen). PCR products were purified using an enzymatic method (ExoSAP-IT; USB Products, High Wycombe, UK) and subjected to sequencing reaction with BigDye Terminater Cycle v1.1 Sequencing Kit (Applied Biosystems). Sequencing reaction products were resolved using a 3100 Genetic Analyzer (Applied Biosystems). Each chromatogram was visually inspected for any abnormalities with particular attention directed to codons 12 and 13.

Results

KRAS mutation analysis by ARMS/S

In a first step one sample per primary tumor (DNA extracted from one tissue core) was tested for *KRAS* mutation with the ARMS/S technology (TheraScreen). *KRAS* mutation was detected in 8/20 (40%) adenocarcinomas and 4/10 (40%) large cell carcinomas. No mutations were found in 10 squamous cell carcinomas.

Screening of intratumoral heterogeneity of KRAS mutation

Between 3 and 8 samples of each primary tumor and up to 4 different lymph node metastases were screened for mutation of *KRAS* codon 12 and 13 by Sanger sequencing. Each sample (DNA extracted from one tissue core) was taken from a different paraffin block to ensure that the samples are from different tumor areas. A total of 181 sequencing reactions were performed for this purpose. Sanger